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Killing bacteria within biofilms by sustained release of tetracycline from triple-layered electrospun micro/nanofibre matrices of polycaprolactone and poly(ethylene-co-vinyl acetate)

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Abstract We report the controlled release of the antibiotic tetracycline (tet) HCl from a triple-layered electrospun matrix consisting of a central layer of poly(ethylene-co-vinyl acetate (PEVA) sandwiched between outer layers of poly-ε-caprolactone (PCL). These micro/nanofibre layers with tet successfully encapsulated (essentially quantitatively at 3% and 5% w/w) in each layer, efficiently inhibited the growth of a panel of bacteria, including clinical isolates, as shown by a modified Kirby-Bauer disc assay. Furthermore, they demonstrated high biological activity in increasingly complex models of biofilm formation (models that are moving closer to the situation in a wound) by stopping biofilm formation, by killing preformed biofilms, and killing mature, dense biofilm colonies of *S. aureus* MRSA252. Tet is clinically useful with potential applications in wound healing and especially in complicated skin and skin-structure infections; electrospinning provides good encapsulation efficiency of tet within PCL/PEVA/PCL polymers in micro/nanofibre layers which display sustained antibiotic release in formulations that are anti-biofilm.

Keywords Biofilms; Drug delivery; Electrospinning; Multilayer; Nanofibre; Polycaprolactone; Poly(ethylene-co-vinyl acetate); Tetracycline; Wound dressing

Introduction

Chronic wounds, such as diabetic ulcers, are hard to heal and require prolonged treatment due to a number of clinical complications, including coagulated proteins and the avascular nature of the eschar around the wound [1, 2]. Consequences of reduced blood irrigation are a restriction of the delivery of the host's immune cells and of systemically administered antibiotics [2]. There has been an increasing interest in the topical application of antimicrobials to overcome the problems associated with the low levels of antibiotic in the granulating tissue [2]. In wound treatment, there may also be a desire to leave dressings on for extended periods, as that will minimise damage to newly formed tissue. However, application of topical antimicrobials requires daily or twice-daily changes of the dressing, leading to patient discomfort as well as being time consuming and costly. In recent years there has therefore been a considerable interest in dressings that allow for a controlled release of antimicrobials [3].

Sadly, infections in chronic wounds are common and they often involve biofilms [4]. That microbial communities exist as biofilms has been demonstrated using light and scanning electron microscopy in 60% of chronic wounds e.g. foot ulcers, pressure ulcers, venous leg ulcers, and ulcerated burn wounds [5, 6]. Biofilms are communities of bacterial cells that are attached to biotic or abiotic surfaces. Cells in these biofilms are enveloped in a matrix of extracellular substance that may include polysaccharides, proteins, and/or DNA, providing protection against different environmental conditions [7-9]. We now know that biofilms are prevalent in nature and indeed appear to be associated with the majority of infections, e.g. wound infections, catheter-linked infections, endocarditis, dental caries, and cystic fibrosis [10]. A particular problem of

bacteria within biofilms is that they are significantly more resistant to antibiotics compared to their free-floating planktonic counterparts [11]. There are several reasons for this, including the slow growth rates of bacteria in biofilms, the reduced penetration of antibiotics (e.g. through production of antibiotic-degrading enzymes), and/or cell density-regulated overproduction of efflux pumps [12]. In addition, cells in biofilms are more resistant to attack by the host's immune system [9], and several pathogens, irrespective of whether they form biofilms or not, have acquired additional resistance mechanisms against antibiotics that are used clinically. Taken together, biofilm-related infections are difficult to treat and eradicate. If left untreated, bacterial infections in wounds may lead to an invasive wound infection and the possibility of sepsis [13]. Thus, it is important to reduce the bioburden in a wound in order to speed up the healing process. In wounds where antibiotic treatment is needed, localized antibiotic delivery systems may overcome the problems associated with the low antibiotic levels in the granulating tissue [2]. As a result of the increased resistance of biofilms to treatment by antibacterial agents, a need has emerged to test the biological activity of antibacterial wound dressings against biofilms rather than against planktonic bacteria. Such models are more relevant to how the bacteria exist in chronic wounds and hence to translational research.

We have recently developed formulations in which tetracycline (tet) HCl has been successfully incorporated in multi-layered electrospun micro/nanofibre matrices of polycaprolactone (PCL) and poly(ethylene-co-vinyl acetate) (PEVA). Its controlled release was demonstrated from the triple-layer (3L) matrix PCL/PEVA/PCL (with 3% w/w tet in each layer, 3L+tet 3%), without loss of chemical stability or bioactivity [14]. The matrices were prepared by electrospinning, a very promising technique for many biomedical applications e.g. tissue engineering and drug delivery systems [15, 16]. The electrospun fibrous matrix was able to sustain the release of tet over more than 15 days [14]. Furthermore, we demonstrated the bioactivity of the released tet against *Staphylococcus aureus* (ATCC 25923) using an

antimicrobial susceptibility test for 5 days [14]. We are therefore working towards establishing that this novel matrix is suitable for applications in wound dressings [17]. In order to make further progress from our fundamental research towards the clinic, it is important to evaluate the effectiveness of our antibiotic formulations against a wider panel of bacteria and with a focus on biofilm-forming *S. aureus* MRSA252. We have verified the activity of our 3L+tet 3% sustained release matrix against five clinical isolates and a further three laboratory strains. The bacteria tested included pathogens that very commonly cause nosocomial infections, such as 4ethicillin-resistant *S. aureus* (MRSA). *S. aureus* is one of the most important pathogens responsible for post-surgical and life-threatening bloodstream infections [18, 19]. In addition, *S. aureus* attaches effectively to both biotic and abiotic surfaces, e.g. catheters and prosthetic joints, and then forms biofilms [20]. We have therefore also investigated a higher concentration 3L+tet 5% sustained release matrix. As highlighted above, chronic wound infections usually involve biofilms rather than planktonic cells, and it is therefore important to test the controlled release matrices against bacterial biofilms. So we also report assays designed to test the levels of antibacterial activity achieved in a series of models of selected wound-associated biofilms of increasing complexity.

Materials

Poly- ϵ -caprolactone (PCL) (Mn 70 000-90 000), poly(ethylene-co-vinyl acetate) (PEVA) (40 wt % vinyl acetate), tetracycline (tet) HCl, and other chemicals, solvents, and membrane filters for epifluorescent microscopy (polycarbonate discs, pore size 0.2 μ m, diameter 13 mm) were purchased from Sigma-Aldrich. Müller-Hinton (MH) agar, MH broth, Tryptone Soya broth (TSB), and antimicrobial tetracycline HCl susceptibility test discs were purchased from Oxoid. Luria-Bertani (LB) broth was purchased from Fisher. Crystal violet 1% was purchased from PRO-LAB Diagnostics. RINZLE (polyvinyl) plastic coverslips were purchased from Electron

Microscopy Sciences. LIVE/DEAD® BacLight Bacterial Viability Kits were purchased from Molecular Probes. Polystyrene 96-well plates (flat bottom, volume 0.36 mL, well diameter 7 mm) were purchased from Costar. Filter discs were cut from Whatman 3MM Chr cellulose chromatography paper (0.34 mm thickness).

Methods

Polymer solutions

PCL was dissolved to a fixed concentration of 12% (w/v) in a 9:1 (v/v) mixture of chloroform (CHCl_3) and methanol (MeOH). Tet was dissolved in the MeOH at 5% of the weight of the polymer. PEVA solution was similarly prepared at 12% (w/v) in $\text{CHCl}_3/\text{MeOH}$ (9:1, v/v) and tet (5% of the weight of PEVA) was likewise incorporated in the MeOH.

Micro/nanofibre fabrication via electrospinning

Each polymer solution was loaded into a syringe and electrospun at 16.5 kV and a flow rate of 1.5 mL/h, with a distance between the tip of the needle and the collector of 13 cm. The flow rate was controlled by a syringe infusion pump (Cole Parmer). The electrospun micro/nanofibrous matrices were collected on two parallel metal electrodes covered with aluminium foil. To fabricate layered mats, each polymer solution was electrospun for a fixed deposition time (40 min) in a layer-by-layer manner. After 20 min, the rectangular collector was rotated through 180° for a second collection of 20 min. Two formulations were designed: a single layer PCL matrix with 5% tet (single layer 5%), and a triple layer PCL/PEVA/PCL matrix with 5% tet in each layer (3L+tet 5%). The 3L+tet 3% matrices were prepared as previously described [14].

Morphology

The surface morphology and cross-sections of the layered electrospun matrices were observed by scanning electron microscopy (SEM). The matrices were cut into small, centimetre-sized pieces, and cross-sections of the layers obtained were freeze fractured in liquid nitrogen. The samples were sputter coated with gold (Edwards Sputter Coater 5150B) and then analysed by SEM (JEOL JSM-6480LV) with an accelerating voltage from 15 kV. The average fibre diameter was determined in layered electrospun matrices containing tet (5%). Some fibre diameter measurements were also taken after drug release (14 days) for comparison. The average fibre diameter was determined by randomly selecting 20 fibres per image from 3 SEM images and measuring their diameters using ImageJ software (NIH, Bethesda, MD; <http://rsb.info.nih.gov/ij/>). The average thickness of each layer was determined by measuring the thickness of the cross-sections of two SEM images using the same software.

Encapsulation efficiency

To determine the encapsulation efficiency of the nanofibres, tet-containing electrospun mats were cut into small pieces ($\sim 1 \text{ cm}^2$), weighed and then dissolved in $\text{CHCl}_3/\text{MeOH}$ (9:1 (v/v), 10 mL). The UV absorbance of each solution was measured at $\lambda = 360 \text{ nm}$, and the amount of tet then determined using a tet calibration curve. Encapsulation efficiency was calculated for each matrix as a percentage of the theoretical maximum tet incorporation (5% w/w in each polymer layer; $n = 3$, in triplicate).

Tet release profile from the triple-layered fibrous matrices

The 3L+tet 3% matrices were cut into 4 cm² squares and each 3% sample was placed in PBS buffer (10 mL; pH = 7.4) in a glass vial. The 5% micro/nanofibrous matrices were punched into circles with diameter of 0.9 cm. Each 5% sample was placed in PBS buffer (5 mL; pH = 7.4) in a plastic vial. The vials were incubated at 37 °C. At specific time intervals (typically 24 h), the buffer (5 or 10 mL) was changed around each matrix sample. The release buffer samples were assayed by UV spectroscopy at $\lambda = 360$ nm and the amount of tet released at each specific time was then calculated using a tet calibration curve and subsequently compared to the theoretical value (3% or 5%) (n = 3, in triplicate).

Bacterial strains and growth conditions

The bacterial strains used in this study were *Bacillus subtilis* 168 [21], *Escherichia coli* NTB5 [22], *Enterococcus faecalis* ATCC 19433 (www.atcc.org), *E. faecalis* BS385 [23], *Pseudomonas aeruginosa* NCTC 6750 (www.hpacultures.org.uk), *P. aeruginosa* PAO1 [24], *Salmonella typhimurium* NTB6 [22], *S. aureus* MRSA252 [25]. All strains were maintained in TSB or LB broth.

Testing the formulations against a panel of bacteria

The strains listed above were used to investigate the efficacy of the 3L+tet 3% matrix and compared to commercially available tet loaded discs and to matrices without tet. For this analysis,

a modified Kirby-Bauer disc assay was used [26]. An overnight culture (16 h) of bacteria was diluted with sterile MH broth to obtain an optical density (OD) of about 0.1 at 600 nm. Then, the diluted bacterial suspension (100 μ L) was spread onto the surface of an MH agar plate and 6 mm circular discs, punched from 3L+tet 3% matrices, were placed on the agar plates. These discs contained 30 μ g tet, which is equivalent to the amount of tet in the commercially available discs. After incubation at 37 °C for 24 h, the growth inhibition zones around the discs were measured.

Antibacterial efficacy of 3L+tet 5% after 14 days of release

S. aureus MRSA252 was used to investigate the efficacy of tet loaded in 3L+tet 5% matrices. For this, a modified Kirby-Bauer disc assay was used. An overnight culture (16 h) of bacteria was diluted with sterile TSB-GN to obtain an optical density (OD) of 0.015 at 600 nm. Then, the diluted bacterial suspension (200 μ L) was spread onto the surface of an MH agar plate and discs, punched from 3L+tet 5% matrices, retrieved following 14 days of tet release, were placed on the agar plates. These discs initially contained ~270 μ g tet, whereas the amount of tet in the commercially available discs was 30 μ g ($n = 3$, in duplicate). After incubation at 37 °C for 24 h, the growth inhibition zones around the discs were measured.

Biofilms studies

96-Well microtiter plate (MTP) biofilm assay

The experiment was carried out as described elsewhere with some minor modifications [22]. *S. aureus* MRSA252 was cultured (15 h) in TSB containing 0.5% glucose and 3% NaCl (TSB-GN) and diluted 20-fold. To investigate the ability of the triple-layer matrix (3L+tet 3% matrix) to

prevent the formation of biofilms, 200 μL /well of the diluted bacterial suspension was dispensed into a polystyrene 96-well plate in 5 groups, with 6 replicates each: a group of matrices (6 mm diameter) containing 30 μg tet (3L+tet 3%), a group of matrices containing no tet (PCL no tet), a group of 30 μg /well tet solution in TSB-GN, a negative control group (media + 30 μg /well tet solution in TSB-GN, without bacteria), and a positive control group containing a diluted bacterial suspension only. The plates were incubated for 24 h at 37 °C on a 3-dimensional plate rotator (40 rpm). The cell suspension was removed and biofilms were washed with sterile PBS (3 x 200 μL /well). The plates were dried for 1 h at 20 °C, and biofilms were then stained with crystal violet solution (1% w/v). After 15 min, the excess of crystal violet and the matrices were removed, plates were washed briefly with water (4 x 250 mL), and the crystal violet was dissolved in aqueous acetic acid (30% v/v in distilled water). The absorbance, representative of the amount of biofilm formed, was measured at $\lambda = 595 \text{ nm}$ (A_{595}).

To test the ability of the triple-layer matrix to eradicate preformed biofilms, 200 μL /well of diluted bacterial suspension (*S. aureus* MRSA252) was dispensed into a polystyrene 96-well plate in 5 groups, with 6 replicates, and the plates were incubated for 24 h at 37°C on a 3-dimensional plate rotator (40 rpm). The cell suspension was removed and biofilms were washed carefully with PBS (1 x 200 μL). Fresh TSB-NG (200 μL) was added to each well, and then matrices or tet solution were added to groups of wells: matrices containing 30 μg tet (3L+tet 3%), matrices containing no tet (PCL no tet), tet solution (30 μg /well), a negative control (media + tet solution 30 μg /well, without bacteria), and a positive control (diluted bacterial suspension only). Plates were incubated again for 24 h at 37 °C, and the biofilms were then washed and stained as above. The amount of biofilm formed was again quantified by measuring the A_{595} .

Killing biofilms by sustained release Tet

To investigate the ability of the matrices to kill formed biofilms in a sustained manner, the discs were introduced onto a preformed biofilm of MRSA252 using the MTP model. The next day, the same discs were transferred to a new biofilm, and this was repeated on day 3. All biofilms were washed and quantified with crystal violet as described before. Further release of tet was analysed with a Kirby-Bauer test by transferring the matrices on day four to an MH agar plate streaked with MRSA252, and this was repeated with the same discs on day five. After incubation at 37 °C for 24 h, the growth inhibition zones around the discs were measured (n = 3, 6 replicates).

Confocal microscopy

The experiment was carried out as described elsewhere, with some minor modifications [27]. *S. aureus* MRSA252 was cultured overnight in TSB-GN, and diluted to an OD at 600 nm of 0.4-0.6. Polyvinyl plastic coverslips (22 mm × 22 mm) were sterilized in absolute isopropanol, dried and placed in 3 wells of a six-well cell culture plate. An aliquot (2 mL) of the diluted bacterial suspension was added to each well and an aliquot (2 mL) of TSB-GN was added. The plate was placed on a 3D rotator (40 rpm) at 37 °C for 24 h, then the media were aspirated off and an aliquot (4 mL) of fresh TSB-GN was added. The 3L+tet 3% matrices were introduced to the wells (20 mm × 20 mm, 20 mm × 20 mm filter paper loaded with an equivalent amount of tet (1.14 mg); one well was left untreated as a control). The plate was incubated at 37 °C for another 24 h, then the media were aspirated off and the coverslips washed with sterile water (6 x 2 mL). To assess the effectiveness of the matrices, coverslips were stained with LIVE/DEAD BacLight mixture (50:50 v/v, 800 µL, 0.3% v/v of each supplied dye solution diluted in sterile water). To prepare the stain from the manufacturer's stock solutions, SYTO® 9 dye solution (3 µL of 3.34 mM in DMSO) was mixed with propidium iodide (3 µL of 20 mM in DMSO) in sterile water (1 mL).

The coverslips were left for 15 min in the dark prior to washing with sterile water (6 x 2 mL). Then the coverslips were mounted on glass slides using nail varnish.

Colony biofilm model (CBM)

The experiment was carried out as described elsewhere, with some minor modifications [28]. *S. aureus* MRSA252 was cultured (15 h) in TSB-GN, and diluted to an OD at 600 nm of 0.4-0.6. Five sterile 13 mm polycarbonate discs were placed on the surface of MH agar plates and aliquots (50 μ L) from the diluted bacterial suspension were spotted on to each disc. Inoculated discs were incubated for 72 h at 37 °C to allow formation of the biofilm. The polycarbonate discs were carefully transferred to new agar plates with sterile forceps on a daily basis. On the fourth day, the discs were covered with 3L+tet 3% or 3L+tet 5% (13 mm diameter, 3% and 5% of the weight of the 3L mat respectively), a filter paper loaded with the same amount of tet as the 3% sample (~390 μ g), and a PCL disc containing no tet (as a negative control). The matrices and the filter paper were cut to 13 mm in diameter; the fifth polycarbonate disc was left without any treatment. Before covering the discs with the 3L matrices, the surface of the biofilms was wetted with MH broth (10 μ L) and, after the coverage, another 25 μ L of broth was applied on top of the matrices. The plates were then incubated again at 37 °C for 24 h and the polycarbonate discs with the matrices were then rinsed gently in sterile 0.9% NaCl to remove any cells that were loosely attached. Each disc was then transferred to a tube containing MH broth (5 mL) and kept cool on ice during the experiment. The tubes were vortexed extensively (~3 min) in order to mechanically disrupt the biofilms and detach the bacteria from the discs. Suspended cells were then serially diluted to 10^{-7} in broth and aliquots (10 μ L) of 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} dilutions were spotted on MH agar plates, to determine the colony forming units (CFU)/mL using the Miles-Misra method [29], as shown diagrammatically in Fig. 1. The plates were incubated at 37 °C for 24 h and the

numbers of CFU were counted. The number of CFU/disc was determined using the following formula:

$\text{CFU/disc} = \text{CFU counted} \times \text{dilution factor} \times 100 \times 5$; this was then normalised by the number of CFU/biofilm disc.

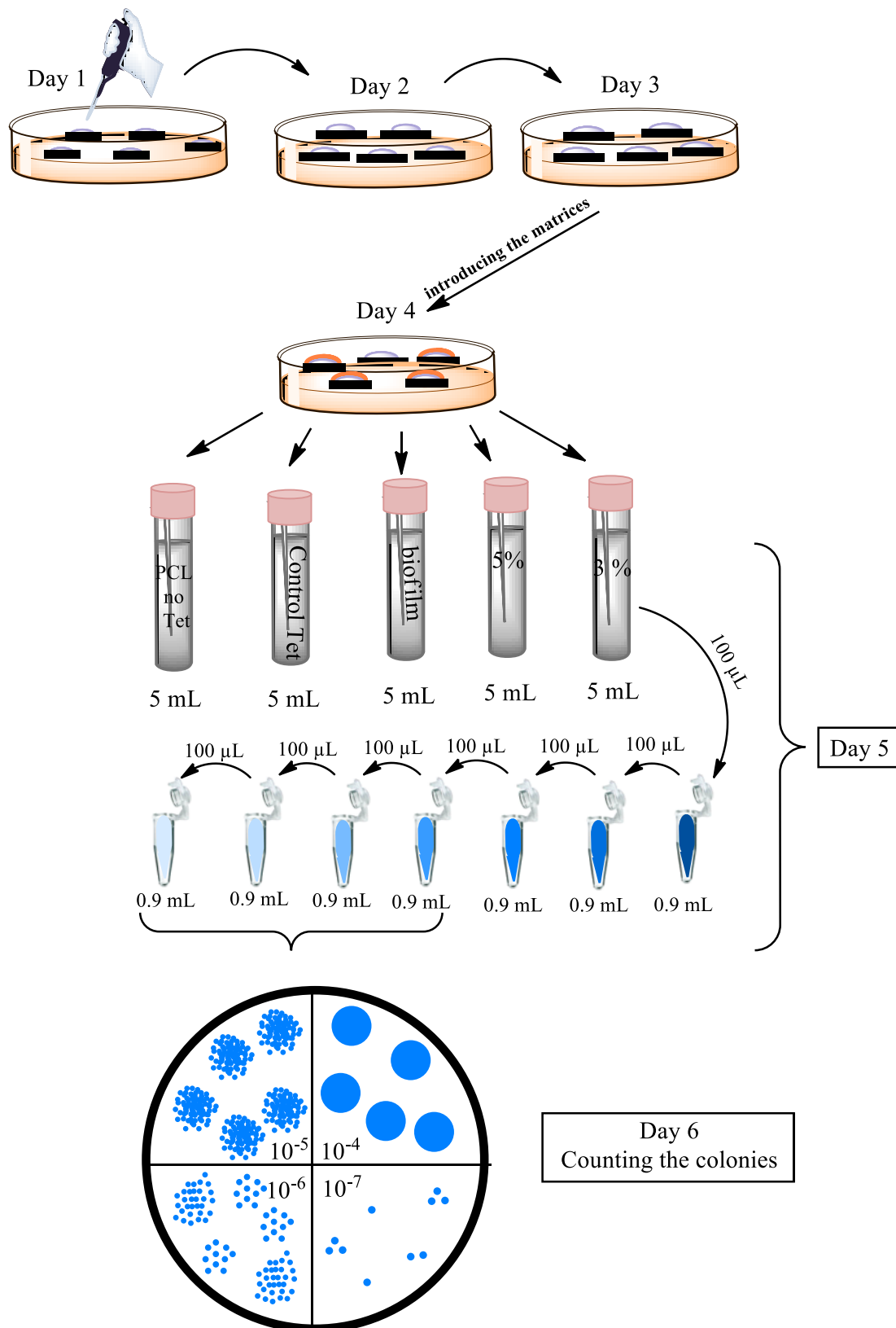


Fig. 1. A sketch describing the colony biofilm model (CBM) experiment.

Results and Discussion

Morphology

The SEM images (Fig. 2) show that the electrospun PCL nanofibres remained intact as the diameters did not change significantly (e.g. 651 ± 251 and 666 ± 255 nm) before and after tet (5%) release (14 days). Electrospun PEVA fibres had a significantly greater diameter (1678 ± 1342 nm) than PCL fibres due to the polymer properties [14]. The cross section of the electrospun 3L+tet 5% matrix was 257 ± 65 μ m.

SIX 6Mb SEM PHOTOS HERE (see other uploaded file)

Fig. 2. SEM images of: a) PCL fibres (*single layer*) electrospun with tet (5%) with diameter of 651 ± 251 nm, b) PCL fibres (*third layer*) electrospun with tet with diameter of 607 ± 336 nm, c) PCL fibres (*outer layer after release of tet for 14 days*) with diameter of 666 ± 255 nm, d) cross section of the PEVA fibres (*second layer*) electrospun with tet with fibre diameter of 1678 ± 1342 nm, e) cross section of the electrospun triple layered matrix electrospun with tet with total thickness of 257 ± 65 μ m, first layer's thickness is 111 ± 18 μ m, f) cross section of second layer with thickness of 57 ± 30 μ m and third layer with thickness of 88 ± 17 μ m.

Encapsulation efficiency

Tet loading in single layer 5% and 3L+tet 5% matrices were $4.76 \pm 0.46 \%$ and $5.3 \pm 0.7 \%$, respectively, compared with 5% (w/w) initial strength. Essentially quantitative drug encapsulation efficiencies of $93 \pm 8 \%$ and $105 \pm 7 \%$, respectively, were achieved.

In vitro tet release studies

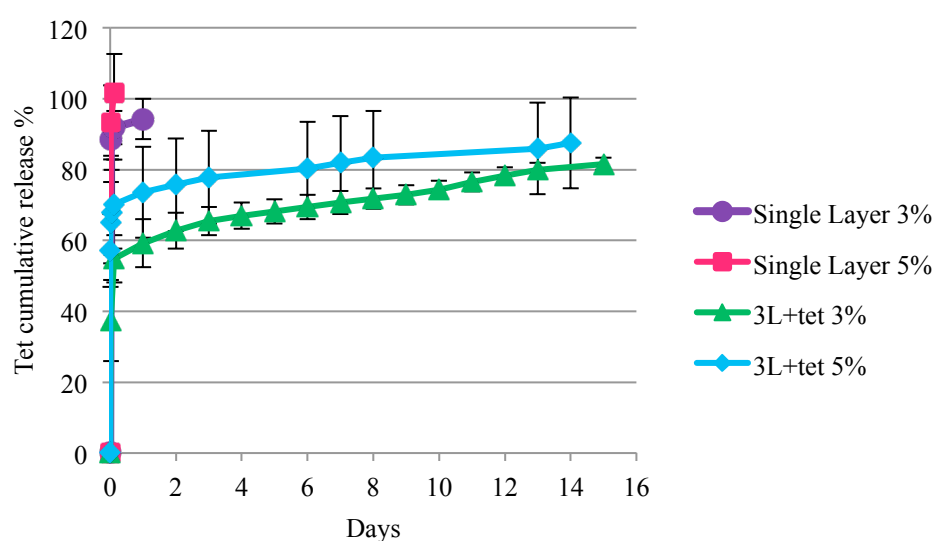


Fig. 3. The tet release profile from the electrospun single- and triple-layered fibrous matrices with two tet concentrations of 3% and 5% w/w. The error bars represent the standard deviation of release values ($n = 3$ in triplicate).

The 3L+tet 3% matrix burst release was 55% of the tet within the first 3 h, then the release of the remaining antibiotic was sustained to reach $\sim 80\%$ at day 14 (Fig. 3) [14]. Likewise, the 3L+tet 5% matrix burst release was 67% of the tet within the first 3 h, then the release of the remaining antibiotic was sustained to reach $\sim 87\%$ at day 14 (Fig. 3). This formulated matrix combines the release profile properties of the two polymers, PCL and PEVA. We have demonstrated that a nanofibrous mat composed only of PCL burst releases essentially all of its content of tet in the

first 30 min, whereas a PEVA only mat sustains the release of tet for 8 days [14]. For a successful antibacterial wound dressing, an initial burst release would help to eliminate any invading bacteria, whereas a sustained release of antibiotic will also aid the immune system of the host in the prevention of any relapse [30].

We have previously reported the antibacterial properties of the 3L+tet 3% matrix against *S. aureus* ATCC 25923 [14]. Here we report similar antibacterial efficacy of 3L+tet 5% matrix after 14 days release demonstrating the sustained release of an efficacious amount of tet from this formulation (Fig. 4). We also report the results of our investigations into the antibacterial properties of this formulation against several bacterial strains. Furthermore, we explore the abilities of the micro/nanofibre matrix to prevent the formation of *S. aureus* biofilms, as well as the ability to eradicate preformed biofilms, a condition which is more closely related to infections in wounds and therefore relevant to translational research of novel wound dressings.

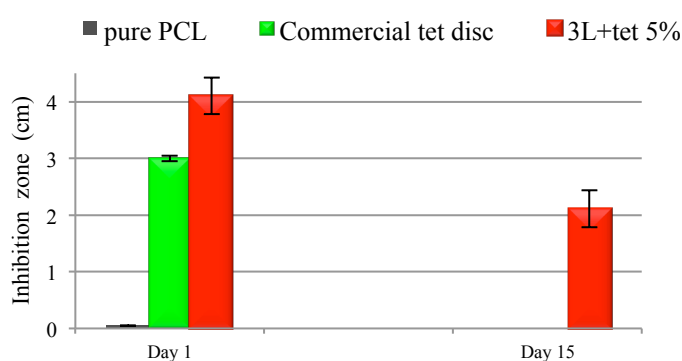


Fig. 4. Diameter of inhibition zone in the MRSA susceptibility test of 3L+tet 5% demonstrating antibacterial efficacy of 3L+tet 5% after 14 days of release. The error bars represent the standard deviation (n = 3, duplicate replicates).

Testing the formulations against a panel of bacteria

Organism	Positive control Zone of inhibition (mm)	3% matrices Zone of inhibition (mm)
<i>B. subtilis</i> 168	32.9 ± 4.1	28.9 ± 5.6
<i>E. coli</i> NTB5	28.1 ± 0.8	24.9 ± 1.4
<i>E. faecalis</i> ATCC 19433	28.2 ± 0.5	28.1 ± 0.9
<i>E. faecalis</i> BS385	22.5 ± 0.8	23.1 ± 1.5
<i>P. aeruginosa</i> NCTC 6750	8.8 ± 0.9	6.7 ± 0.8
<i>P. aeruginosa</i> PA01	11.2 ± 0.2	9.9 ± 1.0
<i>S. aureus</i> MRSA252	36.5 ± 0.7	33 ± 2.4
<i>S. typhimurium</i> NTB6	23.5 ± 0.6	20.9 ± 2.1

Table 1. The antibacterial activity of 3L+tet 3% matrices against a panel of eight bacterial strains.

In total, eight bacterial strains, including three laboratory strains and five clinical isolates, were tested. *B. subtilis* 168 is a very well characterised laboratory strain [21]. The *E. coli* NTB5 and *S. typhimurium* NTB6 strains are clinical isolates obtained from the collection of the Radboud University Nijmegen Medical Centre [22]. *E. faecalis* ATCC 19433 is a commonly used reference strain, whereas *E. faecalis* BS385 is a clinical isolate obtained from biliary stents [23]. Similarly, *P. aeruginosa* NCTC 6750 is a reference strain, whereas *P. aeruginosa* PAO1 is well-characterised clinical strain originally isolated from a wound [24]. Finally, *S. aureus* MRSA252 is a multidrug-resistant clinical isolate that is a representative of an epidemic lineage endemic in UK hospitals. This MRSA is resistant to several antibiotics, including penicillin, ciprofloxacin, erythromycin, and meticillin [25]. With the exception of the *P. aeruginosa* strains, which have a significant level of intrinsic resistance, all strains are sensitive to tetracycline. All strains were

tested against the 3L micro/nanofibre matrices using disc diffusion tests, and compared to commercially available tet discs. As shown in Table 1, there are no significant differences ($P > 0.01$) in the effectiveness of the matrices and in comparison to the commercial tet discs used. Not unexpectedly, the PCL without tet (negative control) showed no effect (data not shown).

Biofilms studies

The different 3L+tet containing matrices were assessed for their efficacy in preventing biofilm formation as well as in eradicating pre-formed biofilms. For the biofilm assays, we used *S. aureus* MRSA252 which is a good biofilm-forming clinical isolate. Initial assessment using the most-commonly used biofilm model, the microtiter plate (MTP) assay. In this assay, bacteria are grown in broth in the wells of a polystyrene 96-well plate. The cells adhere to the bottom of the well and form biofilms there. The amount of biofilm present is assessed using crystal violet staining.

MTP (96-well microtiter plate) biofilm assay

The first MTP assay measured prevention of biofilm growth by *S. aureus* MRSA252. As shown in Fig. 5, at 24 h, tet was indeed efficient in preventing biofilm formation ($A_{595} = 0.11 \pm 0.1$ for biofilms formed in the presence of 3L+tet, versus $A_{595} = 1.80 \pm 0.67$ for biofilms formed in the presence of cells only, $P < 0.01$). There was no significant effect of PCL nanofibres on biofilm formation ($A_{595} = 1.83 \pm 0.62$ for biofilms formed in the presence of PCL no tet versus $A_{595} = 1.80 \pm 0.62$ for biofilms formed in the presence of cells only, $P > 0.01$). Our formulation of 3L+tet micro/nanofibre matrices showed comparable activity to the control ($A_{595} = 0.11 \pm 0.1$ for biofilms formed in the presence of 3L+tet versus $A_{595} = 0.13 \pm 0.06$ for biofilms formed in the presence of tet, $P < 0.01$).

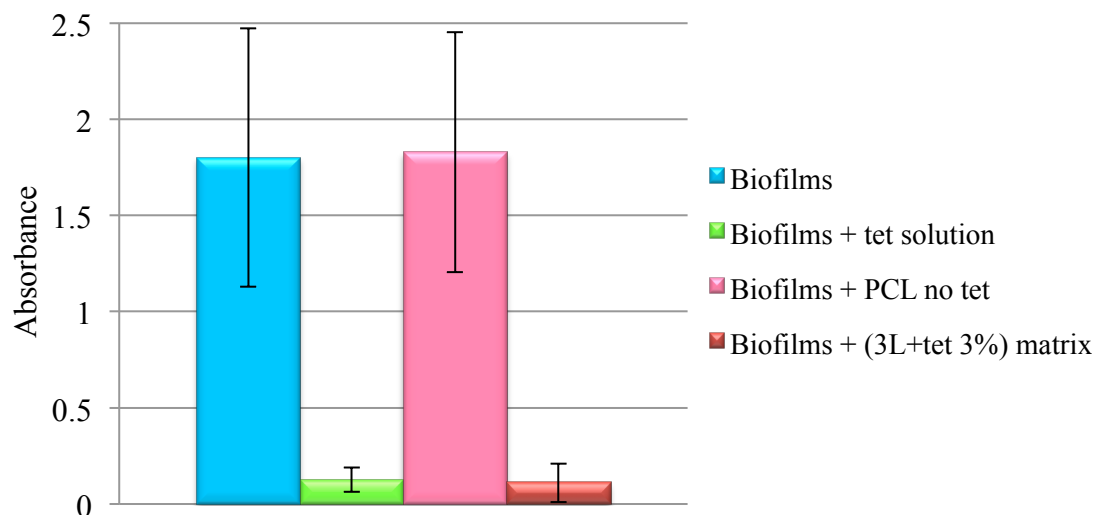


Fig. 5. Prevention of biofilm formation tested using the MTP model. The tet solution is prepared to contain the same amount as the 3L+tet 3% sample. The error bars represent the standard deviation (n = 3, 6 replicates).

It is also important in translational research to investigate the activity of the matrices against preformed biofilms. Infected wounds will typically already contain biofilms, and it is probably also easier to prevent biofilm formation than to kill an existing biofilm. This assessment was done with an MTP assay in which biofilms were first grown for 24 h, after which fresh medium was added to the wells with or without tet matrices. This was followed by another 24 h incubation and then the amount of biofilm was again quantified. As shown in Fig. 6, the 3L+tet matrices showed significant antibacterial activity against *S. aureus* MRSA252 biofilms ($A_{595} = 0.27 \pm 0.13$ for biofilms + 3L+tet versus $A_{595} = 3.49 \pm 1.48$ for biofilms formed in the presence of cells only, $P < 0.01$).

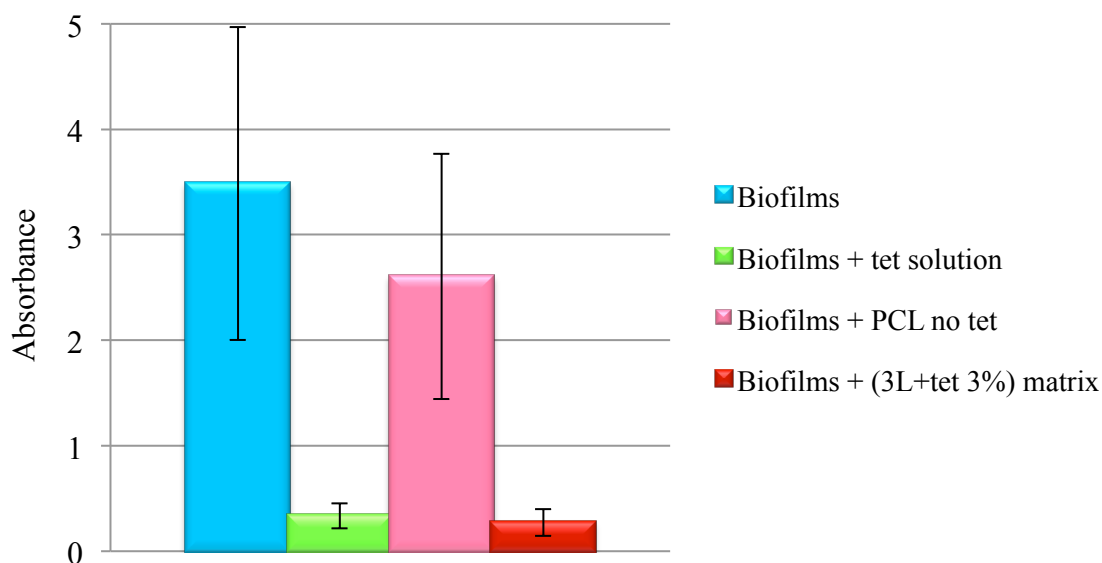


Fig. 6. The activity of the matrices against formed biofilms ($n = 3$) tested using the MTP model. The tet solution is prepared to contain the same amount as the 3L+tet 3% sample. The error bars represent the standard deviation ($n = 3$, 6 replicates).

Killing biofilms by sustained release of tet

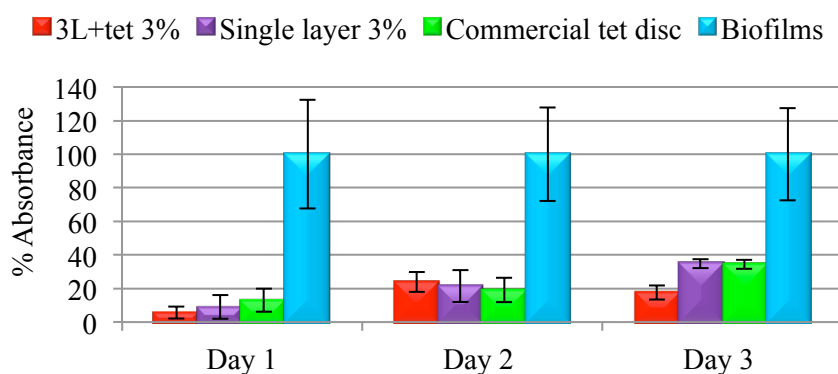


Fig. 7. The activity of the matrices against formed biofilms (normalised absorbance) tested using the MTP model. The error bars represent the standard deviation ($n = 3$, 6 replicates).

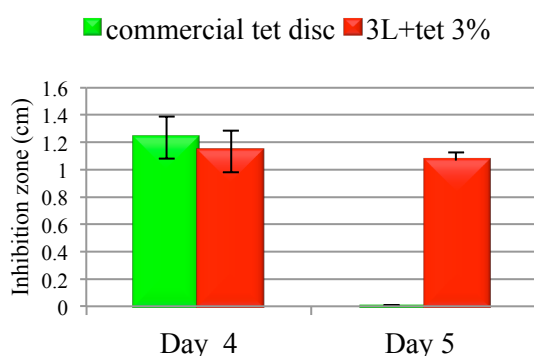


Fig. 8. Diameter of the inhibition zone in the MRSA susceptibility test of 3L+tet 3%. The error bars represent the standard deviation (n = 3, duplicate replicates).

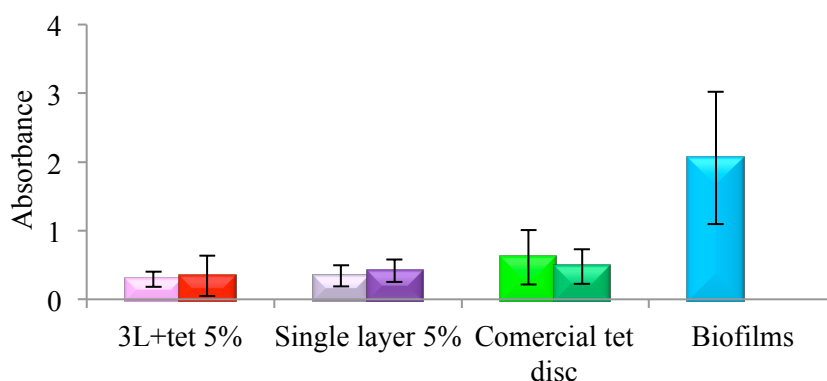


Fig. 9. The activity of the matrices against formed biofilms tested using the MTP model. The error bars represent the standard deviation (n = 3, 3 replicates). The left-hand columns represent the samples where the discs were left in the wells for 3 days, whereas the right-hand columns represent the samples where the discs were left in the wells for 1 day.

In order to test whether the sustained release of tet from 3L+tet 3% was effective against biofilms, discs were placed on preformed MRSA252 biofilms and incubated for 24 h. On day 2, the same discs were then transferred to fresh biofilms, and this was repeated on day 3. All biofilms were quantified with crystal violet. As can be seen in Fig. 7, all discs tested continued to kill preformed biofilms for 3 days. For the first two days there was no significant difference between the different types of discs. However, after 3 days the efficacy of 3L+tet 3% was clearly better than

single layer 3% or commercial tet discs, with 3L+tet 3% killing approximately 84% of the biofilms, whereas the other discs killed around 65% of the MRSA252 biofilms.

The discs that were on biofilms for three consecutive days were then tested for two further days in the Kirby-Bauer disc diffusion assay, again using *S. aureus* MRSA252. On day 4, the zones of inhibition observed for 3L+tet 3% and commercial discs were similar. However, on day 5, when these discs were again transferred to agar freshly inoculated with MRSA252, the commercial tet discs were clearly exhausted as with these only a very small zone of inhibition was observed, whereas the 3L+tet 3% discs still gave rise to zones of inhibition of ~11 mm (Fig. 8). This clearly showed that 3L+tet 3% discs were continuously active over a period of at least 5 days on both biofilms and planktonic cells. The sustained antibiotic activity of the tet (5%) matrices against formed biofilms was further tested using the MTP model. In Fig. 9, the left-hand columns show prevention of growth over 3 days, in comparison with 1 day (the right-hand columns).

Confocal microscopy

To analyse the efficacy of the matrices more qualitatively, biofilms were grown on polyvinyl coverslips in broth and treated both with and without the drug-containing fibrous matrices. The biofilms were then analysed using the LIVE/DEAD BacLight stain which is composed of two fluorescent dyes that stain nucleic acids: SYTO 9 and propidium iodide (Fig. 10). SYTO 9 penetrates bacterial membranes whether they are alive (with intact cell membranes) or dead (having damaged membranes), causing them to fluoresce green. However, propidium iodide, which is at least doubly positively charged, only penetrates bacteria with damaged membranes and its subsequent interaction with nucleic acids results in red fluorescence. When adding SYTO 9 together with propidium iodide both will penetrate dead cells but, due to the higher affinity of propidium iodide for nucleic acids, the cells fluoresce red. Fig. 11 shows the effect of the tet-loaded matrices in killing bacteria even when they are presented in biofilms. The treated

coverslip clearly fluoresces red at a level comparable to that of the tet control, which indicates that the bacteria are dead, whereas the untreated coverslip fluoresces green showing that the bacteria are alive [31].

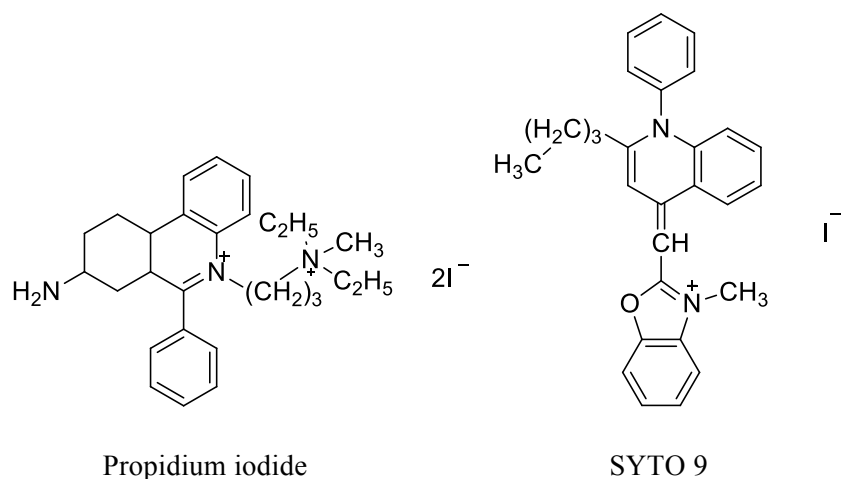


Fig. 10. The two fluorescent dyes in LIVE/DEAD BacLight stain.

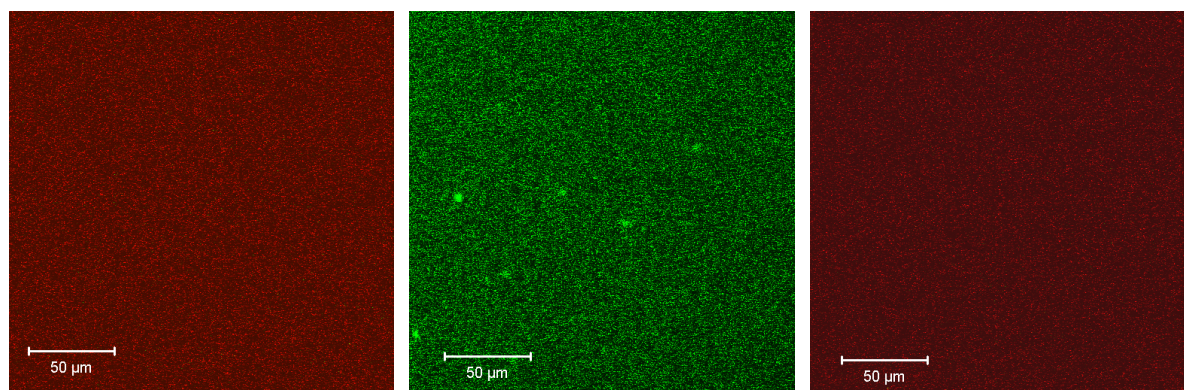


Fig. 11. Confocal microscopy images of biofilms grown on coverslips, from left to right: coverslip treated with 3L+tet 3% matrix, untreated coverslip, coverslip treated with a filter paper + tet loaded with the same amount as 3L+tet 3% matrix as a control.

Colony biofilm model (CBM)

The biofilm model used in the MTP assay is a fairly simple system in which the biofilm grows in liquid broth. A model more closely resembling biofilms growing in a wound is the colony biofilm model (CBM). In this model, biofilms are grown on a polycarbonate membrane that sits on top of an agar plate. This in vitro biofilm model follows from studies by Stewart and co-workers in simulating two aspects of the wound environment: low fluid shear and proximity to an air interface. The CBM captures the geometry of nutrient delivery to a biofilm in a wound in which carbon and nitrogen sources emanate from the host tissue (agar in this model) with oxygen supplied from the opposite side of the biofilm by diffusion into the biofilm from the air interface [28, 32]. This more complex model also has the advantage that it is easy to enumerate the number of live bacteria, as the membrane can simply be taken off the agar plate and cells can then be resuspended in liquid medium. Using this, we investigated the antibacterial activity of the tet matrices against well-developed 72 h-mature biofilms.

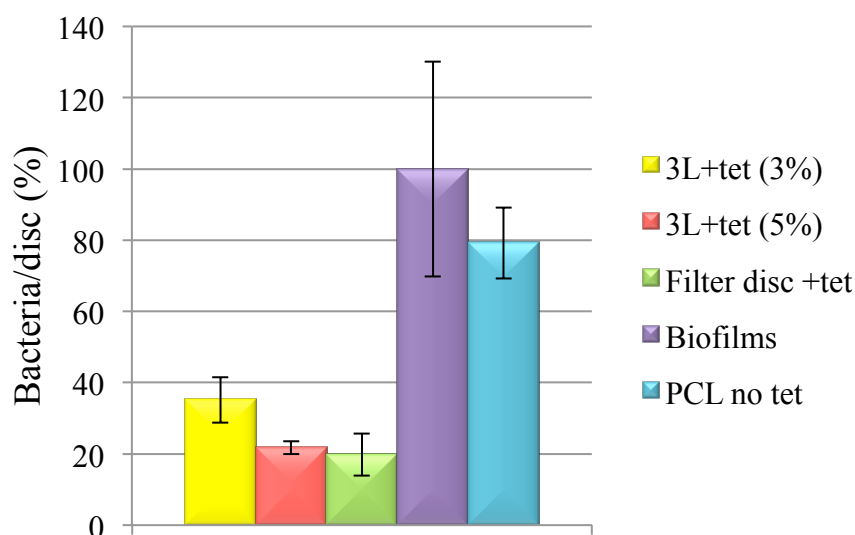


Fig. 12. Effects of the tet (3% and 5%) containing matrices on biofilms grown on polycarbonate discs tested using the CBM model expressed as normalised live bacteria/disc. The filter disc (Whatman 3MM Chr, 0.34 mm thickness) was loaded (by evaporation of a methanolic solution)

with an amount of tet equivalent to the 3L+tet 3% sample loading. The error bars represent the standard deviation ($n = 3$).

Fig. 12 clearly demonstrates the antibacterial effectiveness of the formulations. Compared with the control biofilms, the 3% and 5% tet samples reduced the number of living cells in the biofilms on the polycarbonate discs significantly; the CFU/disc was reduced from 100% for untreated biofilms to 35% for the 3% tet sample ($P < 0.01$). There is a significant further reduction in CFU/disc achieved with the 5% tet sample (down to 21%, $P < 0.01$) compared to that obtained with the 3% tet sample.

One key question was whether the 3L+tet matrix showed reduced efficacy compared to commercial tet discs against a panel of bacteria in the Kirby-Bauer disc diffusion test. Our matrices showed good-excellent antibacterial activity (Table 1). In our experiments, antibacterial susceptibility tests against our panel of different, clinically relevant species of bacteria determined these to be sensitive to tet loaded in the triple-layer fibrous matrix, where our formulation encapsulates a part of the antibiotic in the matrix to achieve initially a burst and then a sustained release which, even after 14 days, was sufficient to kill bacteria (Fig. 4). Although in vitro testing cannot be directly correlated to in vivo performance, in vitro models are valuable as they allow simple, rapid, and inexpensive screening of anti-biofilm agents [33]. Furthermore, improving the efficacy of traditional wound-care products against biofilms will significantly aid in the discovery and selection of novel and appropriate therapies [33].

Several studies have evaluated the effects of wound dressings against both planktonic and biofilm bacterial phenotypes as both of them are present in wounds [34-37], but not all antibacterials that are used clinically and successfully against planktonic bacteria kill biofilms. For example, Percival et al. have observed an enhanced tolerance to silver alginate dressing when a selected number of microorganisms were grown in the biofilm phenotypic state compared to

their non-biofilm counterparts [34, 35]. Hill et al. reported that neither ciprofloxacin nor flucloxacillin could disrupt mixed *Pseudomonas* and *Staphylococcus* biofilms, while only two of six silver-containing dressings showed effects on biofilms grown for 3 days [36]. Hess et al. demonstrated that after incubating *S. aureus* RN6390 and ATCC 25923 biofilms with high concentrations of gentamicin, the *S. aureus* remained viable and biofilm biomass increased [37]. Similarly, we have found that both gentamicin and vancomycin are very inefficient in eradicating preformed *S. aureus* MRSA252 biofilms (data not shown). Here we show that 3L+tet fibrous matrices significantly decrease the biomass of biofilms formed with *S. aureus* MRSA252. This decrease was demonstrated quantifiably using crystal violet in the MTP assay reducing the biomass of a 24 h-biofilm by more than 90%. This was also confirmed qualitatively using the BacLight stain which showed that this 3L+tet fibrous matrix released tet and killed the bacteria rather than dispersing the biofilm. The confocal microscopy images (Fig. 11) provide further support to the MTP assay results that the majority of the bacteria did not survive treatment with the 3L+tet fibrous matrix. In the MTP assay, using the non-specific dye crystal violet, both dead and living cells are stained [38], whereas the BacLight stain distinguishes between live and dead cells [31].

We then examined whether the 3L+tet matrix is capable of eradicating biofilms grown in conditions that more resemble the wound environment. For this we used the CBM assay which is more complex than the MTP assay in that the biofilms grow and mature at an air interface on a polycarbonate disc and nutrients are provided from below [33]. We also investigated a higher concentration (5%) of tet in our sustained release matrices in order to ensure efficacy against biofilms in this more complex model. The CBM model showed that the 3L+tet 5% matrix decreased the viable count in a 72 h-mature biofilm by about 80% (Fig. 12). The residual 20% may be targeted by the host immune system and/or by the sustained released of tet. Bacterial resistance shown in biofilms is mainly caused by the protection afforded by the biofilm to the

bacteria against the host immune system, environmental stress, and antibiotics [4, 5, 33, 39]. For example, Kostenko et al. showed that biofilm bacteria, which survived the initial silver treatment, were susceptible to tobramycin, ciprofloxacin and trimethoprim-sulfamethoxazole, in contrast to untreated biofilms, which were highly tolerant to the same antibiotics [40].

In each of our different in vitro models, with increasing levels of biofilm complexity, the effectiveness of the tet-loaded triple-layer fibrous matrix was similar to the positive tet control, thus even retaining a part of the loaded dose of the tet for sustained release did not lower the initial bacterial killing capability. Using these established models, we have shown that our formulations of tet-loaded triple fibrous matrix are anti-biofilm agents capable of eradicating biofilms made by *S. aureus* MRSA252 a multidrug-resistant clinical isolate that is a representative of an epidemic lineage endemic in UK hospitals.

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Conflict of Interest

All four authors Nour Alhusein, Paul A. De Bank, Ian S. Blagbrough, and Albert Bolhuis declare that they have no conflict of interest. There were no experiments on human or animal subjects.

References

- [1] Moore K, McCallion R, Searle RJ, Stacey MC, Harding KG. Prediction and monitoring the therapeutic response of chronic dermal wounds. *Int Wound J*. 2006;3:89-96.
- [2] Hammond AA, Miller KG, Kruczek CJ, Dertien J, Colmer-Hamood JA, Griswold JA, Horswill AR, Hamood AN. An in vitro biofilm model to examine the effect of antibiotic ointments on biofilms produced by burn wound bacterial isolates. *Burns*. 2011;37:312-321.
- [3] Zilberman M, Kraitzer A, Grinberg O, Elsner JJ. Drug-eluting medical implants. *Handb Exp Pharmacol*. 2010;197:299-341.
- [4] Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW. A review of the scientific evidence for biofilms in wounds. *Wound Rep Reg*. 2012;20:647-657.
- [5] James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS. Biofilms in chronic wounds. *Wound Repair Regen*. 2008;16:37-44.
- [6] Kennedy P, Brammah S, Wills E. Burns, biofilm and a new appraisal of burn wound sepsis. *Burns* 2010;36:49-56.
- [7] Wolcott RD, Dowd S, Kennedy J, Jones CE. Biofilm-based wound care. *Adv Wound Care*. 2008;1:311-316.
- [8] Fux CA, Stoodley P, Hall-Stoodley L, Costerton JW. Bacterial biofilms: a diagnostic and therapeutic challenge. *Expert Rev Anti Infect Ther*. 2003;1:667-683.
- [9] Hornef MW, Wick MJ, Rhen M, Normark S. Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat Immunol*. 2002;3:1033-1040.
- [10] Fux, CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. *Trend Microbiol*. 2005;13:34-40.
- [11] Anwar H, Dasgupta MK, Costerton JW. Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrob Agents Chemother*. 1990;34:2043-2046.
- [12] Høiby N, Bjørnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents*. 2010;35:322-332.

- [13] Church D, Elsayed S, Reid O, Winston B, Lindsay R. Burn wound infections. Clin Microbiol Rev. 2006;19:403-434.
- [14] Alhusein N, Blagbrough IS, De Bank PA. Electrospun matrices for localised controlled drug delivery: release of tetracycline hydrochloride from layers of polycaprolactone and poly(ethylene-co-vinyl acetate). Drug Deliv Transl Res. 2012;2:477-488. doi: 10.1007/s13346-012-0106-y.
- [15] Meinel AJ, Germershaus O, Luhmann T, Merkle HP, Meinel L. Electrospun matrices for localized drug delivery: current technologies and selected biomedical applications. Eur J Pharm Biopharm. 2012;81:1-13.
- [16] Pham QP, Sharma U, Mikos AG. Electrospinning of polymeric nanofibers for tissue engineering applications: a review. Tissue Eng. 2006;12:1197-1211.
- [17] Leaper DJ, Schultz G, Carville K, Fletcher J, Swanson T, Drake R. Extending the TIME concept: what have we learned in the past 10 years? Int Wound J. 2012;9:1-19.
- [18] Kirby JP, Mazuski JE. Prevention of surgical site infection. Surg Clin N Am. 2009;89:365-389.
- [19] National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004. Am J Infect Control. 2004;32:470-484.
- [20] Raad I. Intravascular-catheter-related infections. Lancet. 1998;351:893-898.
- [21] Spizizen, J. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc Natl Acad Sci USA 1958;44:1072-1078.
- [22] Boleij A, Muytjens CM, Bukhari SI, Cayet N, Glaser P, Hermans PW, Swinkels DW, Bolhuis A, Tjalsma H. Novel clues on the specific association of *Streptococcus gallolyticus* subsp *gallolyticus* with colorectal cancer. J Infect Dis. 2011;203:1101-1109.
- [23] van Merode AEJ, van der Klei HC, Buscher HJ, Waar K, Krom BP. *Enterococcus faecalis* strains show culture heterogeneity in cell surface charge. Microbiol. 2006;152:807-814.

- [24] Holloway BW. Genetic recombination in *Pseudomonas aeruginosa*. J Gen Microbiol. 1955;13:572-581.
- [25] Holden MT, Feil EJ, Lindsay JA, Peacock SJ, Day NP, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin R, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth T, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser, H, Holroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Rabinowitsch E, Rutherford K, Sanders M, Sharp S, Simmonds M, Stevens K, Whitehead S, Barrell BG, Spratt, BG, Parkhill J. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci USA. 2004;101:9786-9791.
- [26] Boyle VJ, Fancher ME, Ross RW. Rapid, modified Kirby-Bauer susceptibility test with single, high-concentration antimicrobial discs. Antimicrob Agents Chemother. 1973;3:418-424.
- [27] Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, Heuser J, Dodson KW, Caparon MG, Hultgren SJ. Contribution of autolysin and sortase A during *Enterococcus faecalis* DNA-dependent biofilm development. Infect Immun. 2009;77:3626-3638.
- [28] Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother. 2000;44:1818-1824.
- [29] Miles AA, Misra SS. The estimation of the bactericidal power of the blood. J Hyg (Lond.) 1938;38:732-749.
- [30] Kim K, Luu YK, Chang C, Fang DF, Hsiao BS, Chu B, Hadjiargyrou M. Incorporation and controlled release of a hydrophilic antibiotic using poly(lactide-co-glycolide)-based electrospun nanofibrous scaffolds. J Control Release. 2004;98:47-56.
- [31] Haugland RP. LIVE/DEAD BacLight bacterial viability kits. Handbook of fluorescent probes and research products, 9th edn. Molecular Probes, Eugene, OR, USA. 2002;626-628.

- [32] Folsom JP, Baker B, Stewart PS. In vitro efficacy of bismuth thiols against biofilms formed by bacteria isolated from human chronic wounds. *J Appl Micro*. 2011;111:989-996, doi: 10.1111/j.1365-2672.2011.05110.x.
- [33] James GA, Agostinho AM, Pulcini EDL, In vitro models for the growth and analysis of chronic wound biofilms. *Adv Wound Care*. 2010;1:293-298, doi: 10.1089/awc.2009.0105.
- [34] Percival SL, Slone W, Linton S, Okel T, Corum L, Thomas JG. The antimicrobial efficacy of a silver alginate dressing against a broad spectrum of clinically relevant wound isolates. *Int Wound J*. 2011;8:237-243.
- [35] Percival SL, Thomas JG, Slone W, Linton S, Corum L, Okel T. The efficacy of silver dressings and antibiotics on MRSA and MSSA isolated from burn patients. *Wound Repair Regen*. 2011;19:767-774.
- [36] Hill KE, Malic S, McKee R, Rennison T, Harding KG, Williams DW, Thomas DW. An in vitro model of chronic wound biofilms to test wound dressings and assess antimicrobial susceptibilities. *J Antimicrob Chemother*. 2010;65:1195-1206.
- [37] Hess DJ, Henry-Stanley MJ, Wells CL. Gentamicin promotes *Staphylococcus aureus* biofilms on silk suture. *J Surg Res*. 2011;170:302-308.
- [38] Maroudas NG. Sulphonated polystyrene as an optimal substratum for the adhesion and spreading of mesenchymal cells in monovalent and divalent saline solutions. *J Cell Physiol*. 1977;90:511-519.
- [39] May T, Ito A, Okabe, S. Induction of multidrug resistance mechanism in *Escherichia coli* biofilms by interplay between tetracycline and ampicillin resistance genes. *Antimicrob Agents Chemother*. 2009;53:4628-4639. doi: 10.1128/AAC.00454-09.
- [40] Kostenko V, Lyczak J, Turner K, Martinuzzi RJ. Impact of silver-containing wound dressings on bacterial biofilm viability and susceptibility to antibiotics during prolonged treatment. *Antimicrob Agents Chemother*. 2010;54:5120-5131.